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Description

Background of the inv ntion

With the adv nt of recombinant DNA techn logy, the c ntrolled bacterial production of an enormous variety of useful polypeptides has become possible. Already in hand are bacteria modified by this technology to permit the production of such polypeptide products such as somatostatin (K. Itakura, *et al.*, Science *198*. 1056 [1977]), the (component) A and B chains of human insulin (D.V. Goeddel, *et al.*, Proc Nat'l Acad Sci, USA *76*, 106 [1979]), and human growth hormone (D.V. Goeddel, *et al.*, Nature *281*, 544 [1979]). More recently, recombinant DNA techniques have been used to occasion the bacterial production of thymosin alpha 1, an immune portentiating substance produced by the thymus. Such is the power of the technology that virtually any useful polypeptide can be bacterially produced, putting within reach the controlled manufacture of hormones, enzymes, antibodies, and vaccines against a wide variety of diseases. The cited materials, which describe in greater detail the representative examples referred to above, are incorporated herein by reference, as are other publications referred to *infra*, to illuminate the background of the invention.

The work horse of recombinant DNA technology is the plasmid, a non-chromosomal loop of double-stranded DNA found in bacteria, oftentimes in multiple copies per bacterial cell. Included in the information encoded in the plasmid DNA is that required to reproduce the plasmid in daughter cells (i.e., a "replicon") and ordinarily, one or more selection characteristics, such as resistance to antibiotics, which permit clones of the host cell containing the plasmid of interest to be recoginzed and preferentially grown in selective media. The utility of bacterial plasmids lies in the fact that they can be specifically cleaved by one or another restriction endonuclease or "restriction enzyme", each of which recognizes a different site on the plasmidic DNA. Thereafter heterologous genes or gene fragments may be inserted into the plasmid by endwise joining at the cleavage site or at reconstructed ends adjacent the cleavage site. As used herein, the term "heterologous" refers to a gene not ordinarily found in, or a polypeptide sequence ordinarily not produced by, *E. coli*, whereas the term "homologous" refers to a gene or polypeptide which is produced in wild-type *E. coli*. DNA recombination is performed outside the bacteria, but the resulting "recombinant" plasmid can be introduced into bacteria by a process known as transformation and large quantities of the heterologous gene-containing recombinant plasmid obtained by growing the transformant. Moreover, where the gene is properly inserted with reference to portions of the plasmid which govern the transcription and translation of the encoded DNA m ssage, the resulting expression vehicle can be used to actually produce the p lypeptide s quenc for which the ins rted gen codes, a process referred to as expression.

Expression is initiated in a r gion known as the prom ter which is recognized by and bound by RNA polymerase. In some cases, as in the trp operon discussed *infra*, promoter regions are overlapped by "operator" regions to form a combined promoter-operator. Operators are DNA sequences which are recognized by so-called repressor proteins which serve to regulate the frequency of transcription initiation at a particular promoter. The polymerase travels along the DNA, transcribing the information contained in the coding strand from its 5' to 3' end into messenger RNA which is in turn translated into a polypeptide having the amino acid sequence for which the DNA codes. Each amino acid is encoded by a unique nucleotide triplet or "codon" within what may for present purposes be referred to as the "structural gene", i.e. that part which encodes the amino acid sequence of the expressed product. After binding to the promoter, the RNA polymerase first transcribes nucleotides encoding a ribosome binding site, then a translation initiation or "start" signal (ordinarily ATG, which in the resulting messenger RNA becomes AUG), then the nucleotide codons within the structural gene itself. So-called stop codons are transcribed at the end of the structural gene whereafter the polymerase may form an additional sequence of messenger RNA which, because of the presence of the stop signal, will remain untranslated by the ribosomes. Ribosomes bind to the binding site provided on the messenger RNA, in bacteria ordinarily as the nRNA is being formed, and themselves produce the encoded polypeptide, beginning at the translation start signal and ending at the previously mentioned stop signal. The desired product is produced if the sequences encoding the ribosome binding site are positioned properly with respect to the AUG initiator codon and if all remaining codons follow the initiator codon in phase. The resulting product may be obtained by lysing the host cell and recovering the product by appropriate purification from other bacterial protein.

Polypeptides expressed through the use of recombinant DNA technology may be entirely heterologous, as in the case of the direct expression of human growth hormone, or alternatively may comprise a heterologous polypeptide and, fused thereto, at least a portion of the amino acid sequence of a homologous peptide, as in the case of the production of intermediates for somatostatin and the components of human insulin. In the latter cases, for example, the fused homologous polypeptide comprised a portion of the amino acid sequence for beta galactosidase. In those cases, the intended bioactive product is bioinactivated by the fused, homologous polypeptide until the latter is cleaved away in an extracellular environment. Fusion proteins like those just mentioned can be designed so as to permit highly specific cleavage of the precursor protein fr m the int nded product, as by the action of cyanogen bromide on methionine, or alternativ ly by enzymatic cleavage. S , eg., G.B. Patent Publication No. 2 007 676 A.

The present invention is directed to the creation of expression plasmids for the expression of heterologous genes in bacteria. The procedure of the present invention is illustrated by the construction of an expression vehicle designed for direct expression of heterologous genes from the trp promoter-operator, the illustrated procedure embodying inventions which are the subject of divisional European Applications EP 86548A and EP 154133A.

According to the present invention there is provided a method of creating an expression plasmid for the expression of a heterologous gene which comprises the simultaneous ligation, in phase, of:

(a) a first linear double-stranded DNA fragment containing a replicon and a gene which expresses a selectable characteristic when placed under the direction of a bacterial promoter, said fragment lacking any such promoter, said first fragment having ligatable ends capable of ligating to itself or to fragment (b) or (c);

(b) a second linear double-stranded DNA fragment comprising said heterologous gene, said second fragment having ligatable ends capable of ligating to itself or to fragment (a) or (c); and

(c) a third double-stranded DNA fragment which comprises a bacterial promoter, said third fragment having ligatable ends capable of ligating to itself or to fragment (a) or (b);

the ligatable ends of said fragments being configured so as to be capable of ligating to form a replicable plasmid in which both the gene for the selectable characteristic and the heterologous gene come under the direction of the promoter with the heterologous gene lying transcriptionally downstream of the promoter and upstream of the selectable characteristic gene, the latter being incapable of functional ligation to the promoter fragment other than via fragment (b) wherein the heterologous gene is functionally linked to the promoter, thus permitting use of the selectable characteristic in selection of transformant bacteria colonies capable of expressing the heterologous gene. The selectable characteristic is preferably antibiotic resistance, for example tetracycline resistance. In a preferred embodiment the selectable characteristic is tetracycline resistance and the bacterial promoter is the trp promoter, ligation preferably reconstituting an operon for the expression of ampicillin resistance as well.

The triple ligation of three synthetic DNA fragments, whose ligatable ends are configured so that they can join together only in the desired fashion to create a synthetic gene is known in the prior art (Goeddel et al. Nature *281* (1979) 544—548).

In the accompanying drawings:

Figures 1 and 2 illustrate in successive stages the manner in which an expression plasmid created by the method of the invention to form a system in which other heterologous genes may be interchangeably expressed as fusions with trp E polypeptide sequences.

In the figures, Antibiotic resistance-encoding genes are denoted Ap^R(ampicillin) and Tc^R (tetracycline). The legend "Ap^S" connotes ampicillin sensitivity resulting from deletion f a portion of the gene encoding ampicillin sensitivity. Plasmidic promoters and operators are denoted "p" and "o".

Finally with regard to conventions, the symbol "Δ" connotes a deletion. Thus, for example, reference to a plasmid followed by, say, "ΔEcoRI—XbaI" would describe the plasmid from which the nucleotide sequence between EcoRI and XbaI restriction enzyme sites has been removed by digestion with those enzymes. For convenience, certain deletions are denoted by number. Thus, beginning from the first base pair ("bp") of the EcoRI recognition site which precedes the gene for tetracycline resistance in the parental plasmid pBR322, "Δ1" connotes deletion of bp 1—30 (ie, ΔEcoRI—Hind III) and consequent disenabling of the tetracycline promoter-operator system; "Δ2" connotes deletion of bp 1—375 (ie, ΔEcoRI—BamHI) and consequent removal of both the tetracycline promoter-operator and the structural gene which encodes tetracycline resistance; and "Δ3" would connote deletion of bp 3611—4359 (ie, ΔPstI—EcoRI) and elimination of ampicillin resistance. "Δ4" is used to connete removal of bp ~900—~1500 from the trp operon fragment eliminating the structural gene for the trp D polypeptide.

A more detailed description of the Figure legends, and of the experimental and theoretical background to the work exemplified below, is to be found in the divisional applications (Supra).

Example

Creation of an expression system for trp LE' polypeptide fusions wherein tetracycline resistance is placed under the control of the tryptophan promoter-operator.

The strategy for creation of an expression vehicle capable of receiving a wide variety of heterologous polypeptide genes for expression as trp LE' fusion proteins under the control of the tryptophan operon entailed construction of a plasmid having the following characteristics:

1. Tetracycline resistance which would be lost in the event of the promoter-operator system controlling the genes specifying such resistance was excised.

2. Removing the promoter-operator system that controls tetracycline resistance, and recircularizing by ligation to a heterologous gene and a tryptophan promoter-operator system in proper reading phase with reference thereto, thus restoring tetracyline resistance and accordingly permitting identification of plasmids containing the heterologous gene insert.

In short, and consistent with the nature of the intended inserts, the object was to create a linear piece of DNA having a Pst residue at its 3' end and a Bgl II residue at its 5' end, bounding a gene capable of specifying tetracycline resistance when brought under the control of a promoter-operator system.

Thus, with reference to figure 1, plasmid pBR322

was Hind III digested and the pr truding Hind III ends in turn digested with S1 nuclease. Th S1 nuclease digestion involved treatm nt of 10 μg of Hind III-cleaved pBR322 in 30 μl S1 buff r (0.3 M NaCl, 1 mM $ZnCl_2$, 25 mM sodium acetate, pH 4.5) with 300 units S1 nuclease for 30 minutes at 15°C. The reaction was stopped by the addition of 1 μ1 of 30 × S1 nuclease stop solution (0.8M tris base, 50 mM EDTA). The mixture was phenol extracted, cholorform extracted and ethanol precipitated, then EcoRI digested as previously described and the large fragment *46* obtained by PAGE procedure followed by electroelution. The fragment obtained has a first EcoRI sticky end and a second, blunt end whose coding strand begins with the nucleotide thymidine. As will be subsequently shown, the S1-digested Hind III residue beginning with thymidine can be joined to a Klenow polymerase I-treated Bgl II residue so as to reconstitute the Bgl II restrction site upon ligation.

Plasmid pSom7 Δ2, as prepared in EP154133A was Bgl II digested and the BGI II sticky ends resulting made double stranded with the Klenow polymerase I procedure using all four deoxynucleotide triphosphates. EcoRI cleavage of the resulting product followed by PAGE and electroelution of the small fragment *42* yielded a linear piece of DNA containing the tryptophan promoter-operator and codons of the LE' "proximal" sequence upstream from the BGI II site ("LE'(p)"). The product had an EcoRI end and a blunt end resulting from filling in the BGI II site. However, the BGI II site is reconstituted by ligation of the blunt end of fragment *42* to the blunt end of fragment *46*. Thus, the two fragments were ligated in the presence of T_4 DNA ligase to form the recircularized plasmid pHKY 10 (see Figure 1) which was propagated by transformation into competent *E. coli* strain 294 cells. Tetracycline resistant cells bearing the recombinant plasmid pHKY 10 were grown up, plasmid DNA extracted and digested in turn with Bgl II and Pst followed by isolation by the PAGE procedure and electroelution of the large fragment, a linear piece of DNA having Pst and Bgl II sticky ends. This DNA fragment *49* contains the origin of replication and subsequently proved useful as a first component in the construction of plasmids where both the genes coding for trp LE' polypeptide fusion proteins and the tet resistance gene are controlled by the trp promoter/operator.

Plasmid pSom7 Δ2Δ4, as prepared in EP 154133A, could be manipulated to provide a second component for a system capable of receiving a wide variety of heterologous structural genes. With reference to Figure 2, the plasmid was subjected to partial EcoRI digestion followed by Pst digestion and fragment *51* containing the trp promoter/operator was isolated by the PAGE procedure followed by electroelution. Partial EcoRI digestion was necessary to obtain a fragment which was cleaved adjacent to the 5' end of the somatostatin g ne but not cleaved at the Ec RI site present between the ampicilin resistance g ne and the trp promoter operator. Ampicillin resistance lost by th Pst I cut in the Ap^R gene could be restored upon ligation with fragment *49*.

In a first demonstration the third component, a structural gene for thymosin alpha-one, was obtained by EcoRI and BamHI digestion of plasmid pThα1 (see EP154133A). The fragment, *52*, was purified by PAGE and electroelution.

The three gene fragments *49*, *51* and *52* could now be ligated together in proper orientation, as depicted in Figure 2, to form the plasmid pThα7Δ1Δ4, which could be selected by reason of the restoration of ampicillin and tetracycline resistance. The plasmid, when transformed into E. coli strain 294 and grown up under conditions like those described in Part I, expressed a trp LE' polypeptide fusion protein from which thymosin alpha one could be specifically cleaved by cyanogen bromide treatment. When other heterologous structural genes having EcoRI and BamHI termini were similarly ligated with the pHKY10-derived and pSOM7 Δ2Δ4-derived components, trp LE' polypeptide fusion proteins containing the polypeptides for which those heterologous genes code were likewise efficiently obtained.

Claims

1. A method of creating an expression plasmid for the expression of a heterologous gene which comprises the simultaneous ligation, in phase, of:

(a) a first linear double-stranded DNA fragment containing a replicon and a gene which expresses a selectable characteristic when placed under the direction of a bacterial promoter, said fragment lacking any such promoter, said first fragment having ligatable ends capable of ligating to itself or to fragment (b) or (c);

(b) a second linear double-stranded DNA fragment comprising said heterologous gene, said second fragment having ligatable ends capable of ligating to itself or to fragment (a) or (c); and

(c) a third double-stranded DNA fragment which comprises a bacterial promoter, said third fragment having ligatable ends capable of ligating to itself or to fragment (a) or (b);

the ligatable ends of said fragments being configured so as to be capable of ligating to form a replicable plasmid in which both the gene for the selectable characteristic and the heterologous gene come under the direction of the promoter with the heterologous gene lying transcriptionally downstream of the promoter and upstream of the selectable characteristic gene, the latter being incapable of functional ligation to the promoter fragment other than via fragment (b) wherein the heterologous gene is functionally linked to the promoter, thus permitting use of the selectable characteristic in selection of transformant bacteria colonies capable of expressing the heterologous gene.

2. The method of claim 1 wherein the selectable characteristic is antibiotic resistance.

3. The m thod of claim 2 wherein the s lectable characteristic is tetracycline resistance and

wherein the bacterial promoter is the trp promoter.

4. Th method of claim 3 wherein ligation reconstitutes an operon for the expression of ampicillin resistance as well.

5. A method of any one of the preceding claims, wherein the product of ligation is transformed into bacterial host, and the bacterial host is cultured in a selective medium.

Patentansprüche

1. Ein Verfahren zur Erzeugung eines Expressionsplasmids für die Expression eines heterologen Gens, das die gleichzeige Ligation in Phase

(a) eines ersten linearen doppelstrangigen DNA-Fragments, das ein Replikon und ein Gen enthält, das ein selektierbares Merkmal exprimiert, wenn es der Leitung eines bakteriellen Promotors unterstellt wird, wobei das genannte Fragment einen solchen Promotor nicht aufweist, wobei das erste Fragment ligierbare Enden aufweiste, die fähig sind, an es selbst oder Fragment (b) oder (c) zu ligieren;

(b) eines zweiten linearen doppelstrangigen DNA-Fragments, umfassend das genannte heterologe Gen, wobei das genannte zweite Fragment ligierbare Enden aufweist, die fähig sind, an es selbst oder an Fragment (a) oder (c) zu ligieren; und

(c) eines dritten doppelstrangigen DNA-Fragments, das einen bakteriellen Promotor enthält, wobei das genannte dritte Fragment ligierbare Enden aufweist, die fähig sind, es selbst oder Fragment (a) oder (b) zu ligieren; wobei die ligierbaren Enden der genannten Fragmente so ausgebildet sind, daß sie fähig sind, zu ligieren, um ein replizierbares Plasmid zu bilden, in dem sowohl das Gen für das selektierbare Merkmal als auch das heterologe Gen der Leitung des Promotors unterworfen werden, wobei das heterologe Gen transskriptionell stromabwärts von Promotor und stromaufwärts vom Gen für das selektierbare Merkmal gelegen ist, wobei letzteres Gen zur funktionellen Ligation an das Promotorfragment nur über das Fragment (b) fähig ist, worin das heterologe Gen funktionell an den Promotor gekoppelt ist, wodurch die Verwendung des selektierbaren Merkmals bei der Selektion von transformanten Bakterienkolonien möglich ist, die fähig sind, das heterologe Gen zu exprimieren.

2. Das Verfahren nach Anspruch 1, worin das selektierbare Merkmal Antibiotikaresistenz ist.

3. Das Verfahren nach Anspruch 2, worin das selektierbare Merkmal Tetracyclinresistenz und der bakterielle Promotor der trp-Promotor ist.

4. Das Verfahrn nach Anspruch 3, worin die Ligation außerdem ein Operon für die Expression von Ampicillinresistenz rekonstituiert.

5. Ein Verfahren nach einem der vorhergehenden Ansprüche, worin das Produkt der Ligation in einen bakteriellen Wirt transformiert und der bakterielle Wirt einem selektiv n Medium kultiviert wird.

Revendications

1. Méthode pour la création d'un plasmide d'expression pour l'expression d'un gène hétérologue qui comprend la ligature simultanée, en phase, de:

(a) un premier fragment d'AdN linéaire à deux brins contenant un réplicon et un gène qui exprime une caractéristique pouvant être sélectionnée lorsqu'il est placé sous la direction d'un promoteur bactérien, ledit fragment manquant de ce promoteur, ledit premier fragment ayant des extrémités pouvant être ligaturées, capables de se ligaturer à elles-mêmes ou au fragment (b) ou (c);

(b) un second fragment d'ADN linéaire à deux brins comprenant ledit gène hétérologue, ledit second fragment ayant des extrémités pouvant être ligaturées, capables de se ligaturer à elles-mêmes ou au fragment (a) ou (c);

(c) un troisième fragment d'ADN à dux brins qui comprend un promoteur bactérien, ledit troisième fragment ayant des extrémités pouvant étre ligaturées, capables de se ligaturer à elles-mêmes ou au fragment (a) ou (b);

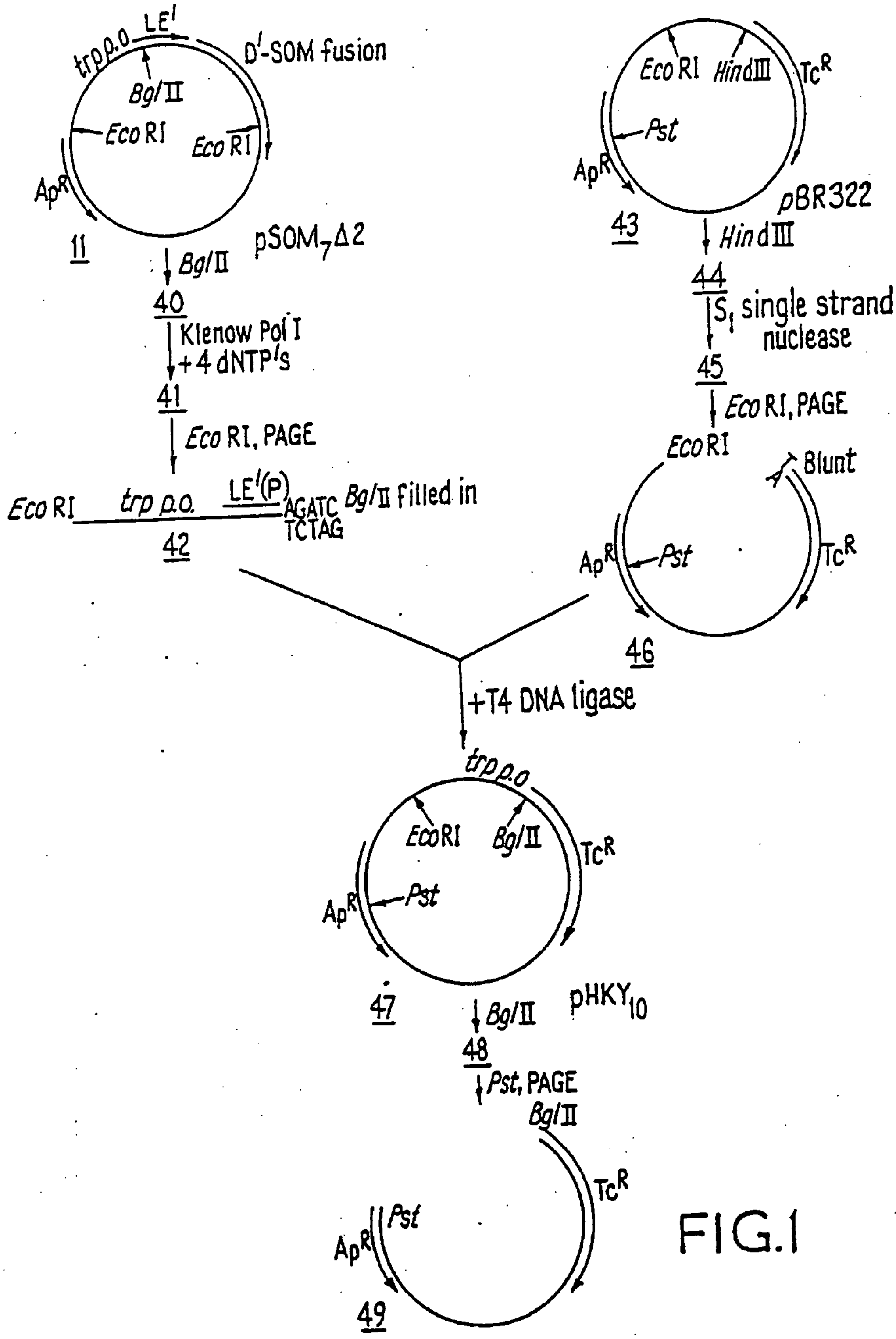
les extrémités desdits fragments pouvant étre ligaturées étant configurées afin d'être capables de se ligaturer pour former un plasmide réplicable où à la fois le gène pour la caractéristique pouvant être sélectionnée et le gène hétérologue viennent sous la direction du promoteur avec le gène hétérologue se trouvant, par transcription, en aval du promoteur et en amont du gène de la caractéristique pouvant ètre sélectionnée, ce dernier étant incapable d'une ligature fonctionnelle au fragment promoteur autre que via l fragment (b) où le gène hétérologue est fonctionnellement liè au promoteur, permettant ainsi l'utilisation de la caractéristique pouvant être sélectionnée, pour la sélection de colonies de bactéries transformantes capables d'exprimer le gène hétérologue.

2. Méthode selon la revendication 1 où la caractéristique pouvant ètre sélectionnée est la résistance aux antibiotiques.

3. Méthode selon la revendication 2 où la caractéristique pouvant être sélectionnée est la résistance à la tétracycline et où le promoteur bactérien est le promoteur trp.

4. Méthode selon la revendication 3 où la ligature reconstitue un opéron pour l'expression de la résistance à l'ampicilline également.

5. Méthode selon l'une quelconque des revendications précédentes où le produit de la ligature est transformé dans un hôte bactérien et l'hôte bactérien est mis en culture dans un milieu sélectif.



trp p.o
LE'
D'-SOM fusion
Bgl II
Eco RI
Eco RI
Ap^R
11
pSOM7Δ2
Bgl II
40
Klenow Pol I
+4 dNTP's
41
Eco RI, PAGE
Eco RI
trp p.o.
LE'(P)
AGATC
TCTAG
Bgl II filled in
42
Eco RI
HindIII
Tc^R
Pst
Ap^R
pBR322
43
HindIII
44
S1 single strand nuclease
45
Eco RI, PAGE
Eco RI
Blunt
Ap^R
Pst
Tc^R
46
+T4 DNA ligase
trp p.o
EcoRI
Bgl II
Tc^R
Ap^R
Pst
47
pHKY10
Bgl II
48
Pst, PAGE
Bgl II
Tc^R
Pst
Ap^R
49

FIG.1

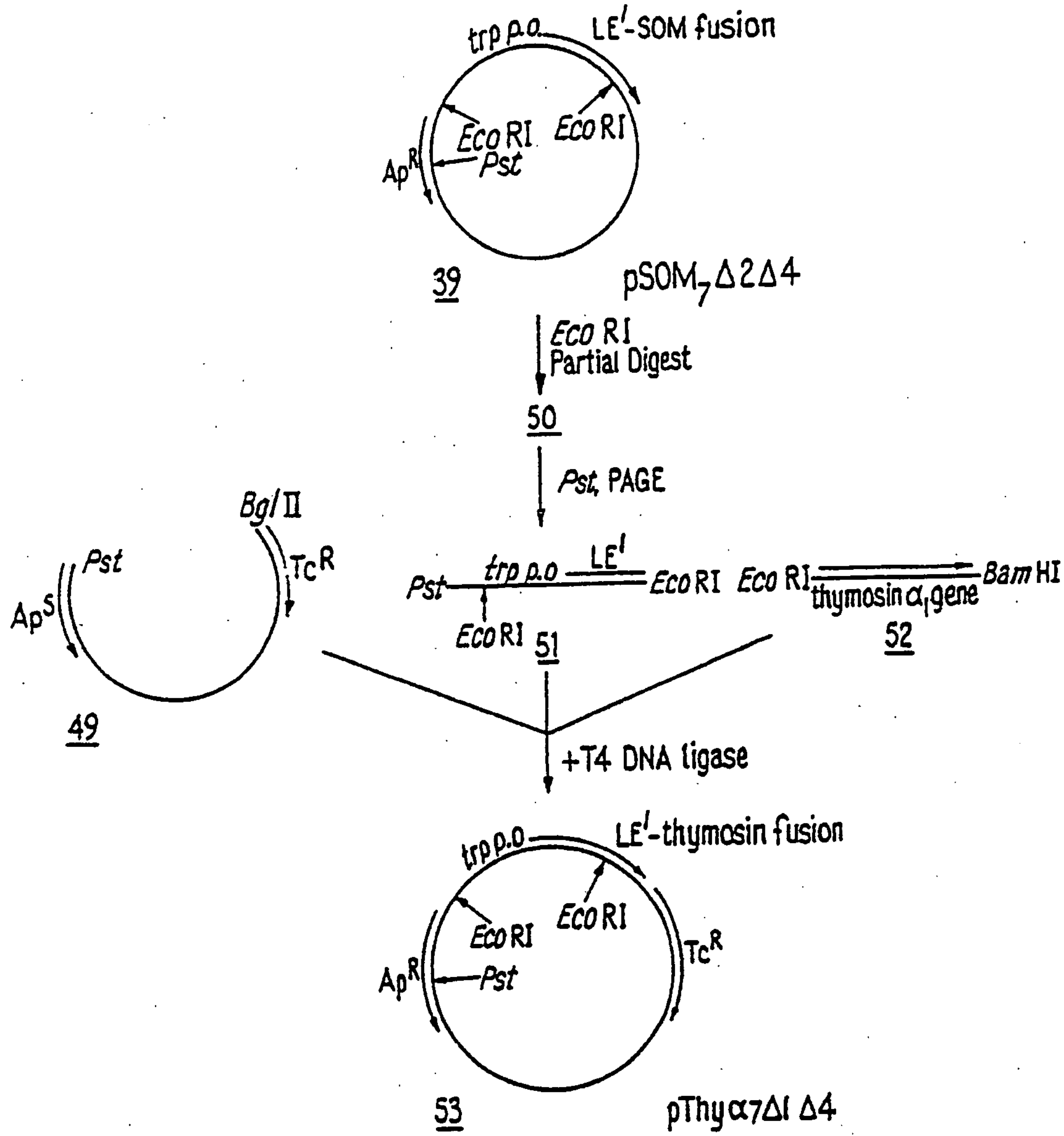

trp p.o.
LE'-SOM fusion
EcoRI
EcoRI
Pst
ApR
39
pSOM7Δ2Δ4
EcoRI
Partial Digest
50
Pst, PAGE
BglII
Pst
TcR
ApS
49
Pst
trp p.o
LE'
EcoRI
EcoRI
51
EcoRI
thymosin α1 gene
BamHI
52
+T4 DNA ligase
trp p.o
LE'-thymosin fusion
EcoRI
EcoRI
Pst
ApR
TcR
53
pThyα7ΔI Δ4

FIG. 2